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Original contribution

Tumor-associated macrophages and the tumor immune microenvironment of primary and recurrent epithelial ovarian cancer☆,☆☆

Laureen S. Ojalvo MD, PhD[a,1], Elizabeth D. Thompson MD[b,c,1], Tian-Li Wang PhD[a,b], Alan K. Meeker PhD[b], Ie-Ming Shih MD, PhD[a,b], Amanda N. Fader MD[a], Ashley Cimino-Mathews MD[b,c], Leisha A. Emens MD, PhD[c,d,*]

[a]*Kelly Gynecologic Oncology Service, Department of Gynecology and Obstetrics, Johns Hopkins Hospital, Baltimore, MD 21287*
[b]*Department of Pathology, Johns Hopkins Hospital, Baltimore, MD 21287*
[c]*Bloomberg-Kimmel Institute at Johns Hopkins, Baltimore, MD 21287*
[d]*Department of Oncology, Johns Hopkins Hospital, Baltimore, MD 21287*

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Summary Tumor-infiltrating lymphocytes (TILs) are associated with better prognosis in newly diagnosed epithelial ovarian cancer (EOC), but clinical trials of immunotherapies in patients with heavily treated disease reveal limited activity. Understanding the tumor microenvironment (TME) of primary and recurrent EOC should guide future trials. Here, we evaluated the TME of paired primary and recurrent tumors (n = 17), and non-paired primary (n = 20) and recurrent (n = 15) tumors, for CD8+ T cells, FOXP3+ regulatory T cells (Tregs), CD68+ tumor-associated macrophages (TAMs), programmed cell death protein 1 (PD-1) and programmed cell death ligand 1 (PD-L1). CD8+ T cells were similar in primary and recurrent tumors, but Tregs were higher in recurrent tumors ($P = .0210$). Higher TAM density (≥5%) associated with higher Tregs ($P = .001$) and CD8+ T cells ($P < .001$) in recurrent tumors, but only with higher Tregs in primary tumors ($P = .02$). TAM-dense recurrent tumors expressed PD-L1 on tumor and immune cells, whereas TAM-dense primary tumors expressed PD-L1 predominantly on immune cells. In survival analyses, higher Tregs in primary tumors correlated with decreased time to first recurrence (17.0 versus 28.5 months, $P = .022$). Conversely, higher Tregs in recurrent tumors correlated with longer overall survival (OS) from recurrence (median not met versus 20.0 months, $P = .022$). TAM density did not affect patient survival. However, patients with increased TAMs at recurrence (n = 5) had longer OS from recurrence compared to patients without increased TAMs (n = 12) (56.0 versus 20.0 months); with the small sample size, this did not reach statistical significance ($P = .074$). Further characterization of the evolution of the TME is warranted.
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* Corresponding author at: Johns Hopkins University School of Medicine, 1650 Orleans Street, Room 409, Cancer Research Building 1, Baltimore, MD 21231.
E-mail address: emensle@jhmi.edu.

[1] Authors contributed equally.

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1. Introduction

Cytotoxic chemotherapy is the mainstay of treatment for women with advanced epithelial ovarian cancer (EOC), but therapeutic failure inevitably occurs [1,2]. This may be due to the emergence of drug-resistant tumor clones due to genomic instability, and/or a tumor microenvironment (TME) that promotes cancer growth [3-5]. Multiple regulatory pathways in the TME support or inhibit the tumor-specific immune response. Antagonists of two inhibitory pathways—specifically the cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed cell death-1 (PD-1) pathways—are now routinely used in the clinic for tumor types other than EOC. In particular, antagonists specific for either PD-1 or PD-L1 have shown activity across multiple solid tumors [6]. Binding of the ligand PD-L1 (present on immune cells and/or tumor cells) to its receptor PD-1 (present on immune cells) prevents immune activation. In clinical trials evaluating PD-1/PD-L1 blockade in ovarian cancer, overall response rates ranged from 11% to 24% [7,8]. Therapeutic strategies to increase the efficacy of immunotherapy are needed in ovarian cancer.

Understanding the milieu of immune cells and immunoregulatory pathways active in the ovarian immune TME should guide the most effective immunotherapy combinations for clinical testing. CD8+ T cells (CD8+ TILs) recognize tumor antigens presented by transformed cells within the TME to mediate tumor cell lysis and disease regression. In advanced disease, both forkhead-box P3 (FOXP3)+ CD4+ regulatory T cells (Tregs) and CD68+ tumor-associated macrophages (TAMs) suppress immunity at the tumor site [9]. The infiltration of Tregs correlates variably with survival in EOC. Prior studies associate Tregs with worse [10,11], improved [12,13] or unchanged [14] impact on survival. However, a high ratio of CD8+ TILs to Tregs correlates with improved median survival in patients with advanced EOC, even after accounting for improved survival in patients with high CD8+ TILs. This illustrates the adverse impact of Treg infiltration and the beneficial effect of CD8+ TILs [14]. High densities of TAMs are more consistently correlated with worse overall survival (OS) in cancer, including EOC [15]. A recent study in high-grade serous carcinoma (HGSC), the most common histologic subtype of EOC [16], identified PD-L1 predominantly on TAMs. Higher PD-L1 expression paradoxically correlated with improved disease-specific survival [17].

Because both cellular composition and immunoregulatory pathway activity evolve with tumor progression, we sought to profile immune cells and immune checkpoint molecules in paired and unpaired primary and recurrent EOC tumor samples.

2. Materials and methods

2.1. Tissue microarrays

This study was approved by the Institutional Review Board (IRB) at the Johns Hopkins Medical Institutions. Four tissue microarrays (TMAs) were constructed from archived paraffin tissue blocks containing primary and recurrent EOCs. Each TMA consisted of 99 spots measuring 1.4 mm in diameter. Three spots per primary and recurrent tumor sample were taken to minimize sampling error. In total, these TMAs included samples from 52 patients: 17 paired primary and recurrent tumors (each pair represented a primary and recurrence from an individual patient), 20 unpaired primary tumors, and 15 unpaired recurrent tumors.

2.2. Database

Approval was obtained from the Johns Hopkins Medical Institutions IRB for the retrospective and prospective collection of demographic information, clinical history, and outcomes data for women with EOC. De-identified patient data were extracted for patients included on the TMAs, and matched to coded histology data for analysis in this study.

2.3. Immunohistochemistry (IHC)

TILs were characterized by IHC labeling for CD8 (mouse monoclonal, clone C8/C8144B, catalogue no. 760-4250, Cell Marque, Rocklin, CA) and FOXP3 (mouse monoclonal, clone 236A/E7, catalogue no. 14-4777-80, eBioscience, San Diego, CA). We manually counted the number of CD8+ (membranous immune cell labeling) and FOXP3+ cells (nuclear immune cell labeling) per one representative high-power field (HPF) for each core, and averaged across cores from the same case of primary or recurrent EOC. Given the lack of standardized cut-off values for immune cell counts, histograms were generated and used to assign high (CD8 $\geq$20, FOXP3 $\geq$10) and low cell counts (Supplementary Figure 1).

TAMs were identified by IHC labeling for CD68 (mouse monoclonal, clone KP-1, catalogue no. 790-2931, Ventana; Tucson, AZ). TAM density was scored by percentage of tumor area (in 5% increments) occupied by cells with diffuse cytoplasmic CD68; cores from the same sample were averaged. TAM density was assigned as none (0%), low (<5% tumor area) or high ($\geq$5% tumor area) given lack of standardized scoring [15].

TMAs were also labeled for PD-1 (mouse monoclonal, clone NAT105, catalogue no. 315M-96, Cell Marque) and PD-L1 (B7-H1) using the rabbit monoclonal anti-human PD-L1 antibody SP142 (Spring Bioscience, Pleasanton, CA) [28]. We scored the percentage of tumor cells with clear membranous PD-L1 labeling in 5% increments, with any intensity labeling >5% considered positive. We scored the percentage PD-L1 labeling by TILs as none (0), focal (1; <5%), moderate (2; 5%–49%), or diffuse (3; 50%–100%). We manually counted the number of PD-1+ (membranous immune cell labeling) per one representative high-power field (HPF) for each core, and averaged across cores from the same case of primary or recurrent EOC. As above, histograms were generated and used to assign high (TIL PD-1$\geq$10) and low counts for PD-1+ cells (Supplementary Figure 1).

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